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Occurrence of a Novel 350-kDa Serine Proteinase in the Fluid of Porcine Ovarian Follicles and Its Increase during Their Maturation

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ABSTRACT—Porcine ovary was found to contain enzyme activities hydrolyzing peptide 4-methylcoumaryl-7-amide (MCA) substrates with a preference for Arg-MCA bond. The activities were shown to be present almost exclusively in the follicular fluid and to increase several times during follicular maturation. The enzyme responsible for these activities is thought to be a serine proteinase as judged from its strong inhibition by diisopropylfluorophosphate (DFP), leupeptin and antipain. The molecular weight of the native enzyme was electrophoretically estimated to be approximately 350,000, the result indicating that the enzyme is clearly distinct from plasmin (M_r=80,000) and collagenase (M_r= 30,000–65,000), both of which are thought to be involved in ovulatory process. The substrate specificity of the partially purified enzyme was qualitatively different from that of plasmin. These results suggest that the enzyme is a novel type of serine proteinase.

INTRODUCTION

The mature ovarian follicle in mammals contains fluid in the follicular space. This follicular fluid is known to consist mainly of transudates of plasma, although it also contains secretory products from follicle cells [1]. Special attention has been paid for proteolytic activities of the fluid in connection with the ovulatory process which is accompanied by drastic degradative changes leading to follicular rupture [2, 3]. At present, collagenase [3, 4] and plasmin [5–8], which is generated from plasminogen by the action of tissue plasminogen activator, are generally thought to be involved in this process.

In an attempt to elucidate the functional role of proteolytic enzymes in mammalian reproductive organs, we happened to find a novel proteinase present in the follicular fluid of porcine ovary, preferable hydrolyzing peptide 4-methylcoumaryl-7-amide (MCA) substrates at Arg-MCA bond. The activity of this proteinase in the fluid was found to increase with follicular maturation. The enzyme is a proteinase with a molecular weight of about 350,000 and is clearly distinct from collagenase and plasmin.

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MATERIALS AND METHODS

Follicular fluid preparation

Porcine ovaries were obtained from Teikoku Hormone Manufacturing Co. (Tokyo, Japan), usually within 4 hours after the animals were slaughtered. Follicular fluid specimens were obtained by aspiration from various stages of follicles using a 21G hypodermic needle and syringe. The samples were centrifuged at 1,000×g for 10 min, and the supernatants were used.

Distribution of follicular fluid enzyme in ovary

Two ovaries were separately placed in a Petri dish containing 5 vol. of cold phosphate-buffered saline (PBS) and gently sliced with a sharp razor blade. The whole materials were collected and

centrifuged at 1,000×*g* for 10 min. The precipitated tissue was gently suspended in 5 vol. of PBS, and the suspension was centrifuged again at 1,000 ×*g* for 10 min. Both supernatants were combined, and this fraction was referred to as "follicular fluid". The tissue fraction was minced with scissors and homogenized in 5 vol. of PBS, and the homogenate was centrifuged at 12,000×*g* for 20 min. The resulting supernatant was referred to as "tissue extract".

Enzyme and protein assays

Peptide substrates containing MCA were obtained from Peptide Institute (Osaka, Japan). Activities were assayed as described previously [9]. Unless otherwise stated, enzyme reactions were conducted in 0.1 M Tris-HCl (pH 8.0) containing 0.1 mM *t*-butyloxycarbonyl (Boc)-Gln-Arg-Arg-MCA and an appropriate amount of sample. Enzyme activity was expressed as the amout of 7-amino-4-methylcoumarin released at 37°C per min.

Protein was determined by the method of Smith *et al.* [10] using the BCA reagent (Pierce).

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) in the absence of sodium dodecyl sulfate (SDS) was performed according to Laemmli [11] with PAG plate 4/15 gradient gel (Daiichi Pure Chemicals Co., Tokyo).

Partial purification of the follicular fluid enzyme

Pig ovary follicular fluid (120 μl) was mixed with an equal volume of the SDS-free sample solvent for PAGE, and the mixture (20 μl) was applied on 12 separate wells. After the run at 4°C, the gel was cut into slices of 2.5 mm width. Each slice was immersed in 2 ml of 0.1 M Tris-HCl buffer (pH 8.0), crushed and left at 4°C overnight. Aliquots of extracts were assayed for enzyme activity. An extract having the highest activity was used as the partially purified enzyme preparation. The sample thus obtained had a specific acitivity of 41.2 nmol of 7-amino-4-methylcoumarin/min/mg protein (Boc-Gln-Arg-Arg-MCA as substrate), which was approximately 64 times greater than that of the crude fluid.

RESULTS

We have found that the crude extract of porcine ovary contains enzyme activities hydrolyzing synthetic, arginine-containing peptide amide substrates. The distribution of the activity in this organ was examined using Boc-Gln-Arg-Arg-MCA as a substrate, and the results are shown in Table I. More than 90% of the total activity was recovered from the follicular fluid, and its specific activity was much higher than that of the tissue extract. The results indicate that the enzyme exists almost exclusively in the follicular fluid.

Figure 1 shows the increase in the activity as the follicles undergo maturation. The specific activities in the follicular fluid preparation derived from follicles with diameters ranging 1–2 mm and 4–5 mm were 0.28 and 0.64 nmol/min/mg protein, respectively. In parallel experiments, the activities toward Ala-MCA (a substrate for aminopeptidase M), Gly-Pro-MCA (asub-strate for dipeptidyl pep-

TABLE 1. Enzyme activity in the tissue extract and follicular fluid of porcine ovary

	Total activity (nmol/min)	Total protein (mg)	Specific activity (nmol/min/mg protein)
Exp. 1			
Tissue extract	4.90	45.0	0.11
Follicular fluid	52.2	108	0.48
Exp. 2			
Tissue extract	2.55	47.8	0.05
Follicular fluid	75.9	124	0.61

The weights of porcine ovaries used were 3.25 g in *Exp. 1* and 3.10 g in *Exp. 2* to obtain the tissue extract and follicular fluid fractions. Detailed procedures are given in MATERIALS AND METHODS.

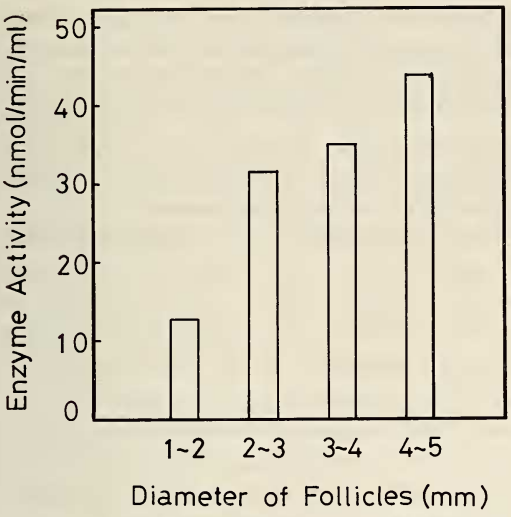

FIG. 1. Change in enzyme activity in follicular fluid preparations during maturation.
The enzyme activity toward Boc-Gln-Arg-Arg-MCA was assayed with the fluids obtained from various stages of ovarian follicles. Follicles with a diameter of 4–5 mm represent those which have almost fully matured.

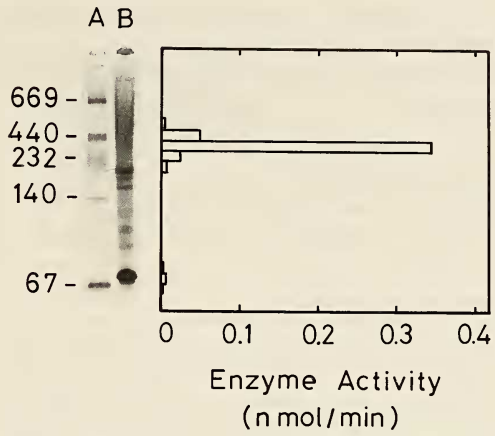

FIG. 2. Electrophoretic analysis of follicular fluid proteins and the enzyme activity.
The fluid preparation from follicles (4–5 mm in diameter) was separately applied on two well positions of a gradient PAGE gel. The protein amounts loaded were 13 μg and 246 μg. After electrophoresis at 4°C, a lane loaded with 13 μg protein was stained with Coomassie Brilliant Blue R-250 (lane B), while the other lane was sliced into pieces of 2.5 mm width for overnight extraction in 0.5 ml of 0.1 M Tris-HCl (pH 8.0) at 4°C. Aliquots of the extracts were assayed for the enzyme activity toward Boc-Gln-Arg-Arg-MCA. The total enzyme activity in each gel slice extract is shown. Lane A shows the separation of molecular weight marker proteins (Pharmacia): thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and albumin (67 kDa).

tidase IV) and N-succinyl-Gly-Pro-MCA (a substrate for prolyl endopeptidase) were also found to be detectable in the fluid. Interestingly, a gradual decline was commonly seen with these enzyme activities during follicular maturation (data not shown). The results strongly suggest that the increase in the activity toward Boc-Gln-Arg-Arg-MCA is rather specific, and is presumably due to accumulation of the corresponding enzyme(s) within ovarian follicles during their maturation.

The electrophoretic analysis of fluid proteins was carried out, and a typical result with the fluid obtained from follicles with a 4–5 mm diameter is shown in Fig. 2. A gel slice extract with the highest activity constituted 82% of the total activity. Figure 2 also shows that the apparent molecular weight of the enzyme is approximately 350,000. When the fluids from less-matured follicles were analyzed under the same conditions, essentially the same patterns were obtained. Thus, we tentatively conclude that an enzyme(s) with M_r= 350,000 is solely responsible for the activity in the fluid that increases during follicular maturation.

Some properties were investigated using the partially purified enzyme sample. The enzyme activity was completely inhibited by DFP (1 mM), leupeptin (20 μM) and antipain (20 μM), whereas E-64 (1 mM), *p*-chloromercuribenzoate (0.23 mM), and *o*-phenanthroline (1 mM) were without effect. These findings are consistent with the classification of the enzyme as a serine proteinase. Table II shows the substrate specificity toward various peptide MCA substrates. The results with porcine plasmin are also included for comparison. The follicular enzyme well hydrolyzed Boc-Gln-Arg-Arg-MCA, Boc-Gln-Gly-Arg-MCA and Boc-Leu-Lys-Arg-MCA, and to some extent Boc-Val-Pro-Arg-MCA. Boc-Glu-Lys-Lys-MCA, a typical substrate for plasmin, was a poor substrate for the enzyme. The effects of the above proteinase inhibitors on the activities toward Boc-Gln-Gly-Arg-MCA, Boc-Leu-Lys-Arg-MCA and Boc-Val-Pro-Arg-MCA were also examined in order to

TABLE 2. Substrate specificity of follicular fluid enzyme

Substrate	Follicular fluid enzyme (%)	Plasmin (%)
Boc-Gln-Arg-Arg-MCA	100	100
Boc-Gln-Gly-Arg-MCA	98.1	9.8
Boc-Leu-Lys-Arg-MCA	112	42.6
Boc-Val-Pro-Arg-MCA	19.6	65.9
Boc-Glu-Lys-Lys-MCA	5.3	91.2

Partially purified follicular fluid enzyme was obtained as described in MATERIALS AND METHODS. For comparison, porcine plasmin (Sigma) was also tested under the same conditions. The plasmin sample had a specific activity of 357 nmol/min/mg protein when assayed with Boc-Gln-Arg-Arg-MCA. The relative values of specific activity are shown.

compare with those toward Boc-Gln-Arg-Arg-MCA. The effects of these inhibitors were very similar to those on the activity toward Boc-Gln-Arg-Arg-MCA (data not shown). Therefore, we presume that these activities are probably catalyzed by a single enzyme, although involvement of several enzymes with very similar properties cannot be ruled out completely at present. The specificity of this follicular fluid enzyme is clearly different from the plasmin specificity.

DISCUSSION

The generation of proteolytic activity within the follicle was suggested years ago as a possible mechanism for degrading the follicular wall [1–3]. Espey *et al.* [3, 4] were able to detect a collagenolytic enzyme in the follicle. On the other hand, Beers *et al.* [5, 6] demonstrated that the proteinase plasmin is capable of weakening follicle wall strips *in vitro*. The two enzyme activities are known to increase in the follicle and reach a peak prior to ovulation. The enzyme described in this study is evidently distinct from these two proteinases as follows: First, as judged from the strong inhibition by DFP, the enzyme is thought to be a serine proteinase, thus being different from a metalloenzyme collagenase. Secondly, the cleavage specificity of the enzyme is qualitatively different from that of a serine proteinase plasmin. Finally, it also differs from plasmin in that the molecular weight of the enzyme is approximately 350,000 while that of plasmin is 80,000. Furthermore, it must be noted that the molecular weight (350,000) is much larger than those observed so far among endoproteinases. Thus, the present enzyme is thought to be a novel serine endoproteinase.

The physiological role of this follicular fluid enzyme is not clear at present. However, our finding that the enzyme activity specifically increases in the fluid as the follicles grow suggests its biological importance in the events relating both with follicular maturation and ovulation. To better understand the detailed molecular and enzymatic properties as well as the physiological role of this enzyme, further studies are necessary including its complete purification and characterization.

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